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Processing and Microfiltration of Mosquitoes for Malaria Antigen Detection in a Rapid Dot Immunobinding Assay

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Data on a technique for the detection of antigen from arthropod vectors in a dot immunobinding assay are presented. In this system, antigen present in the vector was first solubilized in sodium dodecyl sulfate. The homogenate from this process was microfiltered through a two-membrane sandwich; target antigen molecules passed through the first membrane and were immobilized on the second one. The first membrane was nonbinding and served to impinge debris. The second membrane was a high-protein-binding-capacity hydrophobic polyvinylidene difluoride membrane. High signal-to-noise ratios were produced by this method, which is readily adaptable for field use. This assay was used for malaria sporozoites, but it can serve as a general technique that is applicable to other arthropod vectors and etiologic agents.

Enzyme immunoassays are commonly used as diagnostic tests for infectious agents. Numerous microtiter plate- and membrane-based enzyme immunoassays have been formulated for detecting infectious agents in arthropods (9, 10). These assays do, however, have some limitations. Microtiter plate assays may have long incubation times and require special equipment to perform and read the data. Brittle nitrocellulose has been used in membrane-based tests, which can have high backgrounds. There is a need for a simple and rapid technique by which arthropod vectors of disease can be tested for the presence of etiologic agents.

We describe here a general technique for the rapid, direct detection of antigen from arthropod vectors in a dot immunobinding assay. This system is unique in that it involves a two-step process that solubilizes antigen and microfilters debris and immobilizes target molecules onto a solid phase. Arthropod vectors are homogenized in sodium dodecyl sulfate (SDS) and then spot filtered with pressure through a two-membrane sandwich. The first membrane is a nonbinding hydrophilic membrane and serves to exclude debris. The second membrane is a high-protein-binding-capacity hydrophobic polyvinylidene difluoride (PVDF; Immobilon-P; Millipore Corp., Bedford, Mass.) membrane. PVDF has great tensile strength and has been shown to yield high signal-to-noise ratios in other assays (5, 6). By this method, a large amount of antigen is bound to the solid phase for detection by monoclonal antibodies.

This is a practical method for surveying arthropod vector populations and determining risk. It is readily adaptable for field use. Large numbers of samples can be processed quickly and can be assessed by eye with a high sensitivity. The assay described here is for malaria sporozoites, but this technique can be applied to other arthropod-borne agents.

MATERIALS AND METHODS

Antigen and specimens. A recombinant protein, $R32tet_{32}$ (11), was used as antigen in preliminary experiments. $R32tet_{32}$ consists of 32 tetrapeptides from the circumsporozoite gene of *Plasmodium falciparum* ([Asn-Ala-Asn-Pro]$_{15}$ [Asn-Val-Asp-Pro]$_2$) fused to 32 amino acids derived from the tetracycline resistance gene of the plasmid pBR322.

Anopheles stephensi mosquitoes were infected by membrane feeding on cultured *P. falciparum* gametocytes (strain NF-54.3D7) (1). Sporozoites were collected by the method of Ozaki et al. (7). Mosquitoes were washed and decapitated. Heads and bodies were placed in a 0.5-ml centrifuge tube containing a glass wool plug at the bottom. The tube was placed in a 1.5-ml conical tube and spun for 3 min in a microfuge (Beckman Instruments, Inc., Fullerton, Calif.). Sporozoites were passed through the glass wool and were collected in the 1.5-ml tube. Sporozoites were counted with a hemacytometer.

Dot immunobinding assay. A monoclonal murine antibody, designated NFS2 (immunoglobulin G2a [IgG2a]; Y. Charoenvit, personal communication), with specificity for the $R32tet_{32}$ protein of *P. falciparum* was used to determine the presence of circumsporozoite protein on the solid phase. Assay-negative controls were uninfected arthropod homogenates and were used at concentrations equivalent to those of the material tested. Optimal antibody and conjugate dilutions were first determined by block titrations.

Two different solid-phase substrates were used for antigen binding in the assay system: Immobilon-P membrane (hydrophobic PVDF; Millipore Corp.) and a nylon-66 membrane (Zetaprobe; Bio-Rad Laboratories, Richmond, Calif.). Various concentrations of $R32tet_{32}$ were made in sterile distilled H_2O, 0.1% SDS in H_2O, or 0.01% SDS in H_2O. A total of 100 μl was spot filtered through prefilters of a hydrophilic-PVDF membrane (Durapore type HV; pore size, 0.45 μm; Millipore Corp.) or Zetaprobe onto the antigen-binding membranes. All membranes were then allowed to dry at room temperature for 1 h, and nonspecific binding sites were blocked by incubation in a solution of 5% nonfat dry milk in phosphate-buffered saline (PBS)–0.02% Tween 20 for 1 h at room temperature.

An antigen detection assay was performed by incubating membrane strips with the NFS2 monoclonal antibody in PBS–2% nonfat dry milk–0.02% Tween 20 for 1 h. After incubation, membrane strips were washed in PBS–0.02% Tween 20 for 1 min. Membrane strips were then incubated with peroxidase-conjugated anti-mouse IgG (Kirkegaard & Perry Laboratories) for 1 h at room temperature. PVDF strips were washed as described above and placed in a final wash of PBS for 1 min. After this, assay strips were placed in a substrate solution of tetramethylbenzidene (Kirkegaard & Perry), and color development was allowed to continue

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for 5 min at room temperature. All assays were repeated in triplicate. A colorimetric signal was measured by using a white light reflectance densitometer (hand-held, battery operated; model IQ-200; Tobias Associates, Ivyland, Pa.).

Effect of detergent. Antigen was processed before it was spotted onto the membrane by homogenization in SDS-H_2O. Tests were performed with two SDS concentrations (0.01% SDS–H_2O and 0.1% SDS–H_2O) and with H_2O alone. The $R32tet_{32}$ protein was diluted to concentrations ranging from 100 to 0.01 ng/ml before incubation.

Specimen processing. Serial dilutions of *P. falciparum* sporozoites were made in H_2O with 0.01% SDS and ground with a micro-tissue grinder. A total of 100 μl of the resulting mixture was spot filtered, with and without a prefilter of hydrophilic PVDF, onto the Immobilon-P membrane. Concentrations ranged from 5,000 to 10 sporozoites per 1 ml of buffer.

Equivalent concentrations of sporozoites were added to samples of single, uninfected mosquitoes in 1 ml of buffer containing 0.01% SDS and pools of 10 uninfected mosquitoes in 2 ml. This material was homogenized together, using a micro-tissue grinder. A total of 100 μl was spot filtered through the premembrane and the assay membrane, after a brief period of settling (5 min). Membrane spots were approximately 1.6 mm in diameter.

Pools of one laboratory-reared and -infected mosquito with nine uninfected mosquitoes were also made and dissociated in 2 ml of buffer containing 0.01% SDS, as described above. There were an average of 600 sporozoites per mosquito. Dilutions of 1/5, 1/10, 1/50, and 1/100 of the homogenate were then spot filtered through the membranes and assayed as described above.

RESULTS

Spot filtration of $R32tet_{32}$. $R32tet_{32}$ was detected at less than 100 pg/ml in the assay system described here. The signal intensity was lower when protein was applied to the membrane in a diluent with 0.1% SDS. A reduction in the SDS concentration to 0.01% in H_2O resulted in optimal signal-to-noise ratios (Fig. 1).

The signal intensity and limit of sensitivity were greatest when Immobilon-P was used as the solid phase for antigen binding. The signal intensity of $R32tet_{32}$ in a diluent of H_2O spotted onto Immobilon-P with a Zetaprobe membrane prefilter was nearly 100 times less than that with the hydrophilic PVDF membrane prefilter. This was also true when the protein was bound to membrane in a diluent of 0.1% SDS in H_2O. This trend was not as pronounced when the diluent was 0.01% SDS, although the limit of sensitivity when Zetaprobe was used as the premembrane was between 1 and 10 ng/ml versus 10 to 100 pg/ml when hydrophilic PVDF was used as the premembrane.

Protein binding to all premembranes (prefilters) was significant when applied in H_2O. This effect was mitigated at concentrations lower than 1 ng/ml, however. The addition of SDS to a concentration of 0.1% in H_2O lowered the binding of protein to the hydrophilic PVDF premembrane but did not alter binding specificities to the Zetaprobe membrane. A diluent of 0.01% SDS in H_2O was found to be optimal. At that concentration, binding of protein to both premembranes was minimal.

Spot filtration of sporozoites. Serial dilutions *P. falciparum* sporozoites were made in buffer containing 0.01% SDS and were spot filtered through the Immobilon-P membrane. This was done with and without a premembrane of hydrophilic PVDF. Dilutions were made from 500 sporozoites per 100-μl spot to 1 sporozoite per spot. The limit of detection was 5 sporozoites in an unfiltered assay and 10 sporozoites in an assay with the hydrophilic PVDF prefilter. Maximal signal intensity was observed at 500 sporozoites per spot.

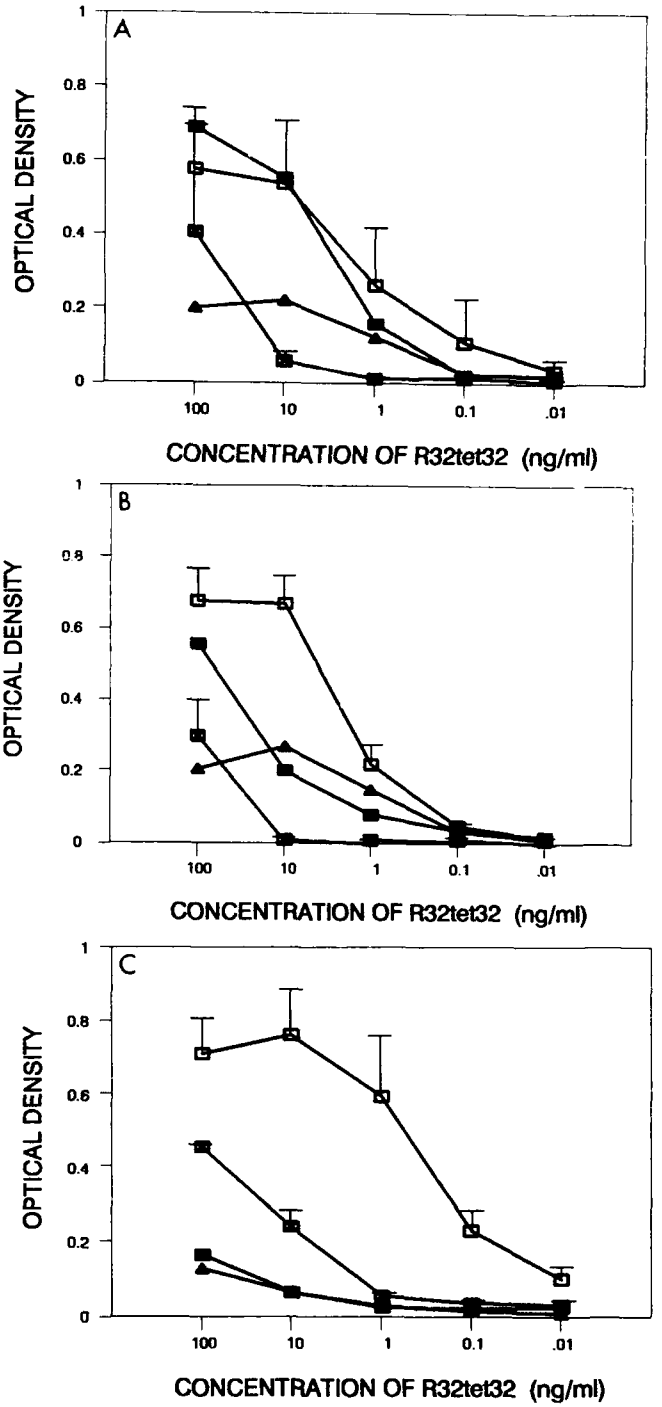

FIG. 1. Comparison of signal intensity of $R32tet_{32}$ spotted through premembranes onto PVDF and Zetaprobe. Symbols: □, Immobilon-P membrane; ⊠, Zetaprobe membrane; ▲, hydrophilic PVDF premembrane; ■, Zetaprobe premembrane. (A) $R32tet_{32}$ in H_2O spotted in concentrations from 100 to 0.01 ng/ml. (B) $R32tet_{32}$ in 0.1% SDS–H_2O. (C) $R32tet_{32}$ in 0.01% SDS–H_2O.

Spot filtration of infected mosquitoes. Serial dilutions, as

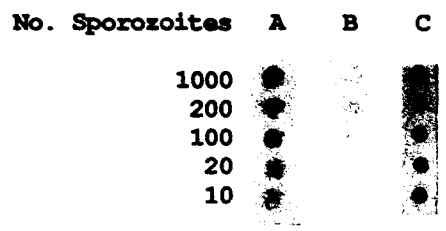

FIG. 2. Spot filtration of mosquito-sporozoite suspensions. (A) PVDF assay membrane showing sensitivity of the process. (B) PVDF assay membrane of negative control test (mosquitoes with no sporozoites). (C) PVDF (hydrophilic) premembrane showing brown pigmented debris.

described above, were added to pools of uninfected mosquitoes. This material was then mixed and homogenized together as described above with 0.01% SDS. A total of 100 μl of the resulting solution was spot filtered through the premembrane and the assay membrane. The addition of 100 sporozoites per 1-ml pool of mosquitoes yielded 10 sporozoites per 100-μl spot. This could be detected on the membrane by eye (Fig. 2). Although a light brown pigment could be seen on the premembrane, none of this coloration was observed on the assay membrane. The limit of detection in the arthropod assay was 10 sporozoites per 1- and 10-mosquito pools.

Pools of one infected mosquito with nine uninfected mosquitoes were made and dissociated in 2 ml of buffer containing 0.01% SDS. Dilutions of 1/5, 1/10, 1/50, and 1/100 of this mixture were then spot filtered through the membranes. As positive signal was observed in dilutions as high as 1/50 and 1/100. It was noted that the color on premembranes, from arthropod bodies, was essentially extinct by a 1/50 dilution.

DISCUSSION

The data presented here illustrate the high signal and low background for the antigen detection assay. This is largely due to the nature of the hydrophobic PVDF membrane (8). Use of a premembrane eliminated any coloration on the assay membrane from arthropod material. In this study, unengorged mosquitoes were tested; therefore, color development caused by substrate reactivity with endogenous peroxidase did not occur. This enzyme activity can be eliminated, if blood meals are present, by incubation of the membrane strips in a dilute hydrogen peroxide solution prior to blocking. Preliminary data indicate that this does not alter assay sensitivity.

Trituration of arthropod vectors in SDS liberates antigen and inactivates enzymes (2–4). Empirical data suggest that SDS also facilitates filtration of antigenic material through the premembrane. This latter point is significant because of the opportunity for impingement of macromolecules on the insoluble debris that is retained in the premembrane. The hydrophilic prefilter excludes arthropod debris but does not bind target antigens. Immobilon-P binds proteins with a high efficiency. This allows sufficient target antigen to be immobilized for detection by monoclonal antibody.

Binding of the $R32tet_{32}$ protein was found to be minimal on the PVDF (hydrophilic) premembrane, in contrast to the binding on other membranes tested. The limit of detection for this protein was equivalent with or without the use of the hydrophilic PVDF premembrane. A concentration of 0.01% SDS in H_2O allowed for the most efficient processing and passage of antigen through the premembrane for binding to Immobilon-P.

The limit of detection for sporozoites alone was lowered from 5 to 10 organisms per 100-μl spot when the hydrophilic PVDF premembrane was used. This was probably due to some amount of macromolecular antigen being impinged. The level of sensitivity for sporozoites in mosquitoes was 10 per pool of 1 or 10 mosquitoes. This was equivalent to the results obtained with sporozoites alone, indicating that there is efficient microfiltration of antigen with minimal impingement on debris.

The sensitivity of this assay and its utility in identifying infected arthropods were very high. Background was essentially nonexistent. The SDS processing-spot filtration technique could be applied, with little or no modification, to the detection of other arthropod-vectored etiologic agents such as *Leishmania* spp. in sand flies and *Borrelia* spp. in ticks.

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